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One series of experiments has studied regeneration of the cut central processes of adult dorsal root ganglion cells into transplants of embryonic central nervous system tissue. Anatomical tracing methods show that cut dorsal roots regenerate into intraspinal transplants of embryonic spinal cord. Immunocytochemical techniques show that the regenerated dorsal roots contain calcitonin gene-related peptide (CGRP) and that many of these axons establish synapses within transplants that resemble those formed in the normal spinal cord. Cut dorsal roots also regenerate into intraspinal transplants of embryonic brain, but growth into embryonic spinal cord transplants is more robust. Another series of experiments has used immunocytochemical, in situ hybridization, and cell counting methods to show that cutting the peripheral processes of dorsal root ganglion neurons has profound effects on tachykinin synthesis and can cause the cells to die, whereas the neurons survive axotomy of their central processes and tachykinin synthesis is unaffected.

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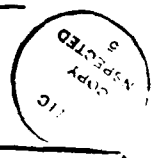

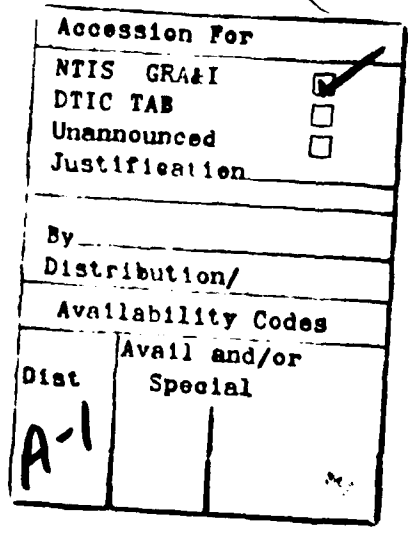

## TABLE OF CONTENTS

## MID-TERM REPORT

## (5) INTRODUCTION

Transplants of embryonic CNS tissue provide a powerful experimental approach to understanding the features of the neural environment that interact with damaged neurons to support or enhance regeneration. Experiments over the past 15 years, primarily involving areas of brain, have demonstrated that neurons thought to be incapable of growth (1) can not only regenerate if supplied with a transplant of fetal CNS but also form connections that are functional by electrophysiological and behavioral criteria (reviewed in 3; 21). Although the mechanisms by which transplants restore function remain to be clarified, they provide both an experimental approach to understanding repair mechanisms and a potential therapeutic strategy (8).

The spinal cord has been a technically more challenging site in which to obtain survival of transplants, but embryonic CNS tissue taken from areas of brain (5; 6; 18) as well as spinal cord (2; 16; 20; 23) grows and integrates with the host. The extent to which these transplants support regeneration of adult host intraspinal axons, however, is uncertain. It is also unclear whether axons that regenerate into intraspinal transplants retain the ability to synthesize normal proteins. The central processes of dorsal root ganglion (DRG) neurons provide an attractive model system in which to study these questions. First, it is well known that the cut central processes of adult DRG neurons regenerate along the dorsal root, but fail to traverse the dorsal root entry zone and re-enter the spinal cord (4; 19). Second, horseradish peroxidase (HRP) transport methods can be used to identify dorsal root axons unambiguously within spinal cord or transplants. Third, DRG axons can be classified into subsets based on the presence of neuropeptides such as calcitonin gene-related peptide (CGRP) (7), and immunocytochemical methods can be used to identify these axons and therefore to determine whether or not the peptides are present in transplants. Because of these advantages, we proposed to use the adult DRG-fetal transplant model to study several features of axon growth into transplants (Specific Aim 3). We first used HRP transport methods and CGRP immunocytochemistry to show that cut dorsal root axons regenerate into transplants of embryonic spinal cord and that some of these axons contain CGRP (23).

To determine the functional potential of these regenerated dorsal root axons, it is important to establish whether or not they make synaptic contacts with neurons within the transplants. We therefore used HRP tracing methods and immunocytochemical techniques to show that regenerated dorsal roots form synapses within transplants of embryonic spinal cord and that these synapses resemble those formed in the dorsal horn of normal spinal cord. Since the environment within which regenerated axons form synapses may also contribute to their functional potential, we used stereological methods to examine in detail the composition of transplanted spinal cord and compared it to the structures found in normal dorsal horn (14).

It is unknown whether the properties of the transplants that allow regenerating axons to grow and establish synapses are specific to the normal target of the cut axons, or if they are common to embryonic CNS tissue generally. The early outgrowth of developing axons is thought to depend on signals that are expressed generally throughout the embryonic CNS, whereas growth within a target and synapse formation appear to rely on more specific cues (reviewed in 17). The requirements of regenerating axons both for early outgrowth (22; 9) and for synapse formation (24) may differ from those of developing axons but have received relatively little attention. We therefore used quantitative morphological methods to investigate whether or not regenerating adult dorsal root axons can grow into and establish synapses in transplants of embryonic brain regions that are not their normal targets and whether the patterns of growth and synapse formation differ in transplants of spinal cord and brain. In this way we distinguish characteristics of growth that are target-specific from those that are shared by non-target embryonic CNS tissue (15).

If the therapeutic potential of transplants is to be realized, it is important to understand the factors that contribute to the survival of transplanted neurons. DRG transplants provide advantages for understanding this problem, and Specific Aims 1 and 2 proposed to use DRG transplants to study factors that contribute to the survival of transplanted neurons and axon growth. We have carried out several studies to provide background information for these experiments. First, we compared the effect on survival of cutting the central or peripheral processes of newborn DRG neurons and also compared DRG neuron survival after cutting the sciatic nerve in adult or newborn rats (13). Second, we used immunocytochemical and histochemical methods to determine whether or not specific populations of DRG neurons are particularly likely to die after axotomy (12). Third, we used immunocytochemistry and in situ histochemistry to examine in detail the metabolic response to axotomy of tachykinin-containing DRG neurons (10; 11).

(6) **BODY**

a. Methods

1. Surgical Methods. Female Sprague-Dawley rats (200-300g) are deeply anesthetized and undergo a laminectomy to expose the fourth lumbar (L4) segment. The adjacent dorsal roots are cut near the dorsal root entry zone and reflected caudally. A hemisection cavity 3-4mm in length is aspirated from the lumbar enlargement, the appropriate transplant is introduced into the cavity, and the L4 or L5 dorsal root stump is juxtaposed to the transplant. The surgical wound is then closed in layers. The surgical techniques have been described in detail in publications from this laboratory, as have the techniques for neural graft preparation (14; 23, included in Appendix). For sciatic nerve section, the right sciatic nerve of deeply anesthetized adult or newborn rats is sectioned in the mid-thigh (see 13).

2. Labeling Methods.

i. Dorsal root labeling. Dorsal roots entering the transplants are labeled with 10% HRP and 1% WGA-HRP (wheat-germ agglutinin-conjugated HRP) as described (14; 23; see Appendix).

ii. Sciatic nerve labeling. The sciatic nerve ipsilateral to the transplant is labeled with an intraneural injection of 0.75% cholera toxin-conjugated HRP or 2% WGA-HRP (14).

iii. CGRP and tachykinin immunocytochemistry. Sections are prepared for LM and EM examination with the PAP technique (14).

iv. In situ hybridization. These methods are described in publications from this laboratory (9; 10).

3. Stereological and morphometric analyses. These methods are detailed in 14.

b. **Results**

1. Adult dorsal root axons regenerate into transplants of embryonic spinal cord. *(This manuscript has been published and is in the Appendix. Tessler, A., B.T. Himes, J. Houle, and P.J. Reier. 1988. Regeneration of adult dorsal root axons into transplants of embryonic spinal cord. J. Comp. Neurol. 270:537-548.)* Anatomical tracing methods that use transport or diffusion of HRP demonstrate that severed host dorsal roots grow into transplants of embryonic day 14 (E14) spinal cord (Fig. 1a). Most of these regenerated axons remain within 2mm of the interface with the host dorsal root, but some axons penetrate as far as 3mm. Many more regenerated axons are demonstrated with immunocytochemical methods that visualize CGRP (Fig. 1b). The results of this study therefore show that transplants of embryonic spinal cord support or enhance the regeneration of cut adult host DRG axons and that many of those that regenerate continue to synthesize peptides that they make normally.

2. Regenerated DRG axons form synapses in transplants of embryonic spinal cord. *(This manuscript has been published and is in the Appendix. Itoh, Y. and A. Tessler. 1990. Ultrastructural organization of regenerated adult dorsal root axons within transplants of fetal spinal cord. J. Comp. Neurol. 292:396-411.)*

Labeling methods that use diffusion of WGA-HRP (Fig. 2a) and CGRP immunocytochemistry (Fig. 2b,c) show that regenerated DRG axons establish synaptic contacts with neurons in embryonic spinal cord transplants. As in normal dorsal horn, the majority of the CGRP-labeled axon terminals are axodendritic, but a large number are also axosomatic and axoaxonic. Regenerated dorsal root axon terminals in transplants are significantly larger than those found in normal dorsal horn, and their synaptic contact length is also increased, suggesting that a compensatory mechanism for increasing synaptic efficacy might occur within transplants. The results of this study show that regenerated dorsal root axons form synapses within transplants and that these synapses retain characteristics of those found in normal spinal cord. These results therefore encourage the expectation that transplants may one day be used to restore neural circuits damaged by trauma or disease.

3. Cut DRG axons regenerate into transplants of brain and form synapses there. *(This manuscript is about to be submitted to J. Comp. Neurol. An abstract has been published: Itoh, Y., C. Rogahn and A. Tessler. 1989. Adult dorsal root axons regenerate into intraspinal transplants of fetal spinal cord (SC) more readily than into fetal brain transplants. Soc. Neurosci. Abst. 15 (Part 1):321.)*

We used immunocytochemical methods for labeling CGRP to examine whether cut dorsal root axons regenerate into and establish transplants in embryonic brain transplants and to compare the patterns of growth in brain and spinal cord transplants. Transplants of embryonic hippocampus, cerebellum, and neocortex and embryonic spinal cord were studied. CGRP-immunoreactive axons regenerate into all of the brain regions and form synapses in the neocortex and cerebellum transplants in which they were sought. Synapses are far rarer than we have observed in solid spinal cord transplants, and the patterns of ingrowth differ in transplants of brain and spinal cord. Both the area fraction and area density occupied by regenerated axons in spinal cord transplants are significantly larger than in neocortex or cerebellum transplants (Table 1). In addition, the distribution of regenerated axons within spinal cord transplants is heterogeneous, since areas of either dense or sparse ingrowth are observed, whereas the distribution in transplants of brain is homogeneous but sparse. Several measurements of the extent of axon distribution, however, including area, longest axis, and length of lateral extension, indicate that CGRP-labeled axons spread more widely in neocortex transplants than in solid transplants of spinal cord or cerebellum (Table 2). These results indicate that embryonic CNS tissues that are not normal targets support or enhance the growth of severed dorsal roots and suggest that the conditions that constitute a permissive environment for regenerating axons are relatively non-specific. Embryonic spinal cord, the normal target of dorsal roots, appears to supply additional more specific cues that enable regenerating axons to grow and arborize within the transplant and to establish relatively normal numbers of synapses. These cues appear to depend at least in part on the integrity of transplant structure, since growth into solid transplants of spinal cord exceeds growth into cell suspensions.

4. DRG neurons die after sciatic nerve section. *(An article and an abstract have been published on this subject: Himes, B.T. and A. Tessler (1989) Death of some dorsal root ganglion neurons and plasticity of others following sciatic nerve section in adult and neonatal rats. J. Comp. Neurol. 284:215-230; Himes, B.T., C. Rogahn, and A. Tessler (1989) Effects of neonatal axotomy on DRG neurons: an immuno-and histochemical analysis of three populations. Soc. Neurosci. Abst. 15 (Part 1):444).*

We used immunohistochemical and histochemical methods along with cell counting techniques to demonstrate that DRG neurons die after sectioning their

peripheral processes and to show that several subsets of DRG neurons are equally likely to die after this injury. DRG neurons do not die after section of their central process, suggesting that death is the result of causes other than axotomy alone.

5. Changes in tachykinin synthesis after axotomy. *(One article has been published and another has been submitted for publication: Henken, D.B., A. Tessler, et al. (1988) In situ hybridization of mRNA for beta-preprotachykinin and presomatostatin in adult rat dorsal root ganglia: comparison with immunocytochemical localization. J. Neurocytol. 17:671-681; Henken, D.B., A. Tessler, et al., (1990) Expression of beta-preprotachykinin mRNA and tachykinins in rat dorsal root ganglion cells following peripheral or central axotomy. Submitted to Neuroscience).*

We used in situ hybridization and immunocytochemical methods to show that peripheral axotomy but not central axotomy causes reduced synthesis of the mRNAs that encode tachykinins and the tachykinins themselves. Changes in synthesis therefore are due to mechanisms other than a nonspecific response to injury, such as reduced levels of Nerve Growth Factor (NGF) which is derived from the peripheral but not the central target of DRG neurons.

## (7) CONCLUSIONS

It follows from this work that cut dorsal roots of adult DRG neurons regenerate into transplants of embryonic spinal cord, whereas they do not regenerate into spinal cord in the absence of a transplant. The regenerated dorsal roots contain peptides found in normal dorsal roots, and they establish synapses that resemble those found in normal spinal cord. These findings are encouraging for the hope that transplants may one day serve a therapeutic function and contribute to the restoration of damaged neural circuits. The stimulus that elicits or supports dorsal root regeneration appears not to be specific to embryonic spinal cord, the normal target of dorsal roots, because dorsal roots also regenerate into transplants of embryonic brain. Regeneration into brain regions is less robust than into spinal cord, however, and synapses are far less frequent, suggesting that regenerating adult neurons require specific cues for growth within a target and synapse formation. Whether or not various subsets of DRG neurons regenerate with equal vigor remains to be determined. Cutting their peripheral process produces profound metabolic changes in several subsets of DRG neurons and can cause the cells to die, whereas the neurons survive central axotomy and changes in tachykinin synthesis are not observed. The conditions that favor the survival of DRG neurons with combined axotomy of central and peripheral processes also remain to be determined.

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## (9) APPENDIX

Copies of the following articles and abstracts which have been prepared during the current period of grant support.

### Publications

Reier, P., J.D. Houle, L. Jakeman, D. Winialski and A. Tessler. 1988. Transplantation of fetal spinal cord tissue into acute and chronic hemisection and contusion lesions of the adult rat spinal cord. Prog. Br. Res. 78:173-179.

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Itoh, Y. and A. Tessler. 1989. Adult dorsal root axons regenerate into intraspinal transplants of fetal spinal cord (FSC) more readily than into fetal brain transplants. Soc. Neurosci. Abst. 15:321.

## TABLE 1. Comparison of Area Occupied by CGRP-labeled Axons

| Group | | N | Area[1] ($\times 10^4 \mu m^2$) | Area density[2] |
|---|---|---|---|---|
| E14 spinal cord | (A) | 5 | 5.63 ± 0.35 | 9.51 ± 1.03 |
| E14 spinal cord-DCS[3] | (B) | 5 | 3.99 ± 0.43 | 4.64 ± 0.66 |
| E18 occipital cortex | (C) | 5 | 2.98 ± 0 46 | 1.64 ± 0.12 |
| E15 cerebellum | (D) | 5 | 1.01 ± 0.12 | 2.66 ± 0.54 |
| Significant differences among groups[4] : | | | Area : A > B, C > D | |
| | | | Area density : A > B, D, C | |

Mean ± S E M. are given.
Area occupied by CGRP-labeled axons is calculated by point-counting stereological analysis.
[2]*Area occupied by CGRP-labeled axons per unit area* ($100 \mu m^2$) *of* CGRP-innervated regions
[3]DCS dissociated cell suspensions
[4]Overall significance determined by one way ANOVA ($p<0.05$) and individual post-hoc comparisons are with Duncan's multiple range test *corrected for multiple comparisons* ($p<0.0001$).

## TABLE 2. Comparison of Distribution of CGRP-labeled Axons

| Group | | N | Distribution[1] Area ($mm^2$) | Distribution[1] Longest axis (mm) | Lateral extension (mm) |
|---|---|---|---|---|---|
| E14 spinal cord | (A) | 5 | 0.62 ± 0.06 | 1.81 ± 0.20 | 1.14 ± 0.06 |
| E14 spinal cord-DCS[2] | (B) | 5 | 0.96 ± 0.17 | 1.66 ± 0.22 | 1.38 ± 0.01 |
| E18 occipital cortex | (C) | 5 | 1.82 ± 0.22 | 2.34 ± 0.13 | 1.45 ± 0.07 |
| E15 cerebellum | (D) | 5 | 0.41 ± 0.06 | 1.27 ± 0.11 | 0.99 ± 0.10 |
| Significant differences among groups[3] : | | | Area[4] : C > B, A, D[4] | | |
| | | | Longest axis : C > A, B, D[5] | | |
| | | | Lateral extension : C, B > A, D[6] | | |

Mean ± S E M are given.
[1]The distribution of CGRP-immunoreactive axons in the sagittal plane is determind by making montages which consist of all the individual sampling lattices examined.
[2]DCS dissociated cell suspensions
[3]Overall significance determined by one way ANOVA ($p<0.05$) and individual post-hoc comparisons are with Duncan's multiple range test corrected for multiple comparisons at the [4]$p<0.0001$, [5]$p<0.001$, and [6]$p<0.05$ levels.


**Figure 1** - Sagittal sections of embryonic spinal cord grafts 1 month after transplantation. Bar - 100 μm. a: Host dorsal root (DR) axons labeled with 2% WGA-HRP regenerate into the transplant (TP) and arborize within the graft. Axons show numerous varicosities and some end in globular dilatations (arrows). b: CGRP-immunoreactive axons are shown in host dorsal root (DR) and transplant (TP). Regenerated axons contain varicosities along their paths and are tangled together to form dense plexuses (*). Inset shows relationship of transplant (TP) and host spinal cord (H) and the interface (arrowheads) between dorsal root (DR) and transplant. Stained with chromoxane cyanine R and cresyl violet (Clark, '81). Bar - 500 μm.

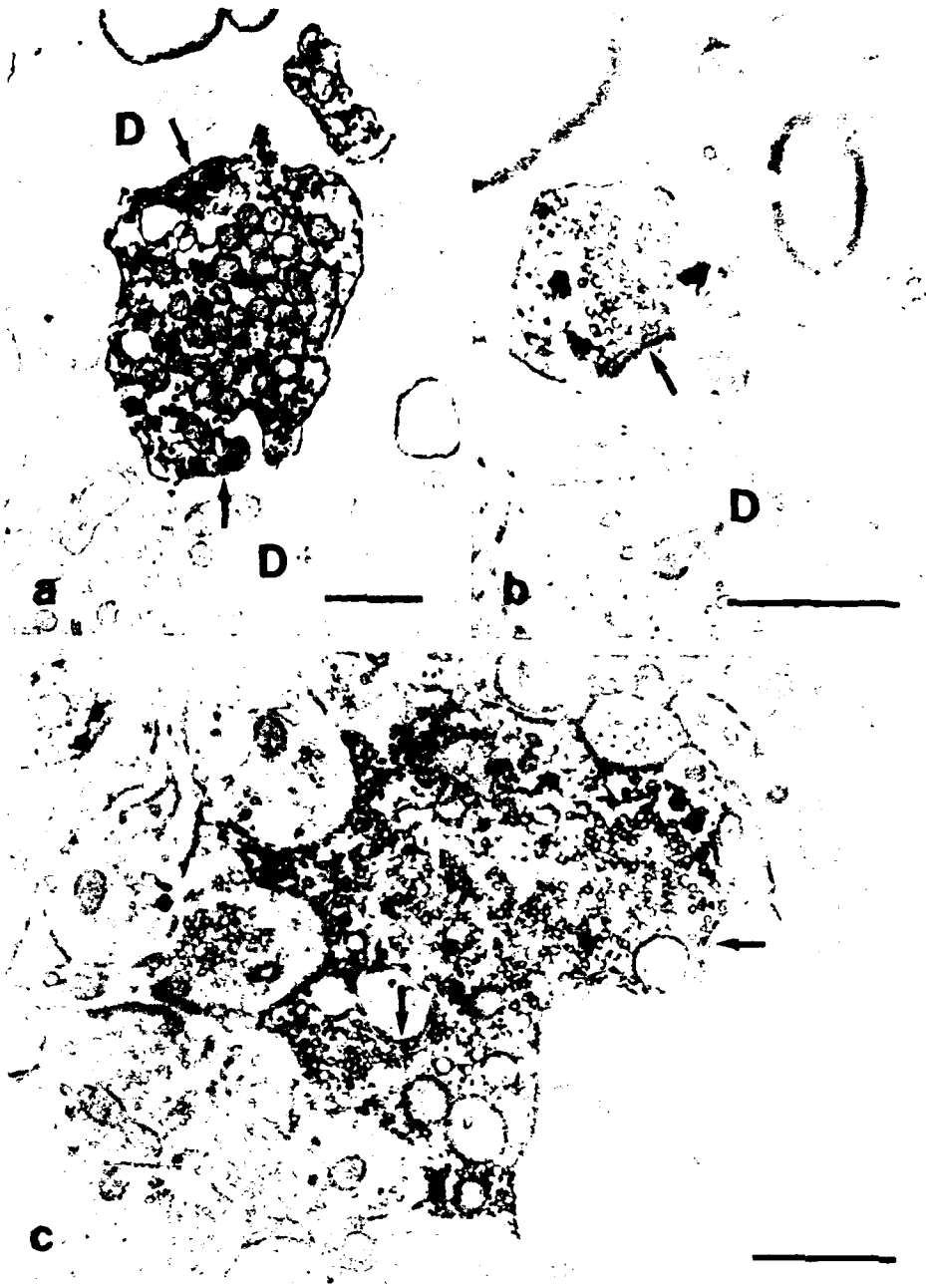


**Figure 2** - Electron micrographs from transplants. Bar - 1 μm. a: A WGA-HRP-labeled complex presynaptic terminal containing spherical vesicles makes asymmetric synaptic contacts (arrows) upon different dendritic profiles (D). b: A typical asymmetric axodendritic synapse (arrow) in which a CGRP-labeled presynaptic profile contacts a dendrite (D). c: An example of a CGRP-immunoreactive complex terminal containing spherical and dense-cored vesicles that makes asymmetric synaptic contacts (arrows) upon different dendritic profiles. This terminal is surrounded by numerous vesicle-containing profiles.